THE SEPARATION OF SPECIES OF MICROSPORIDA USING SEROLOGY, MICROELECTROPHORESIS AND ISOELECTRIC FOCUSING

By

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Abstract of Dissertation Presented to the Graduate Council
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Four biochemical techniques, serology, isoelectric focusing, and microelectrophoresis of extracts of disrupted and intact spores were used to separate species of Microsporida. Serological (Ouchterlony) techniques successfully separated the 6 Microsporidans tested although some cross reaction was seen between 3 of them. Antigens displayed slight differences caused by variations in host rearing temperature but were not effected when spores were aged for 5 months. Isoelectric focusing successfully separated 7 Microsporidans but indicated a close relationship between 5 of them. Isoelectric focusing spectra were effected by variations in host rearing temperature and spore age but were stable when the same Microsporidan was raised in 2 different hosts.

Three Microsporidans were separated using microelectrophoresis of protein extracts of disrupted spores. However, great difficulty was encountered in removing polyacrylamide gels from the tubes, possibly the result of poor polymerization. The technique needs additional work before results may be relied on. Five Microsporidans were separated using extracts from intact spores (Spore coat protein). Fewer bands were

detected in this method than were seen from disrupted spores but these present were species specific.

CHAPTER 1

INTRODUCTION

The Microsporida, a previously obscure group of obligate intracellular Protozoan pathogens of arthropods and fish, have received considerably more attention of late due to their potential value as biological control agents and their effect on economically important organisms. As a result, the present taxonomic system which is based on the work of Leger and Hesse (1922) has been found to be inadequate. Recent revisions by Sprague (in press) and Hazard and Oldacre (in press) have helped to clarify the situation but the taxonomy of this group is still in a state of flux.

Whether new or old, the taxonomy of the Microsporidia is based solely on morphology. Many authors, however, have found that classical morphological features such as spore size and shape and host specificity are of dubious value in differentiating species (Fowler, 1971). Modern techniques such as electron microscopy have proved to be of considerable value but even with this instrument species separation and determination of taxonomic relationships may be difficult. Clearly there is room for a biochemical approach to microsporidian taxonomy.

Biochemical studies have been hindered by difficulties involved in disrupting the spores which are the most readily available stage of the life cycle. Canner (1970), however, found that the MSK Braun cell homogenizer provides good disruption. Fowler and Reeves (1974a,b) analyzed protein liberated from disrupted spores using disc gel electro-

pheresis and were able to separate some microsporidian isolates at the species level. The present study was intended to be an extension of the electrophoresis work of Fowler and Reaves (1974a) and an investigation of the applicability of two other protein separation techniques, isoelectric focusing and serology to the field of microsporidian taxonomy.

Materials and Methods

Spore Production

Spores were grown in 3 lepidopteran hosts, corn earworms (Heliothis zea), Indian meal moths (Plodia interpunctella) and cabbage loopers (Trichoplusia ni). All larvae were reared in a temperature controlled room at 27°C with 70% relative humidity.

Corn earworm eggs were obtained from the Southern Grain Insects Research Lab (A.R.S., U.S.D.A.) at Tifton, Ga. After hatching, the worms were reared for 4 days in individual cups according to the method of Burton (1969). Worms were infected with isolates 942, 954 and 960 (Table 1) by placing a few drops of a suspension containing 10^5-10^6 spores/ml on the surface of the medium. Those to be infected with isolates 650, 906, 907, and 1116 were starved for 24 hours before 2 drops of a spore suspension containing 10^5-10^6 spores/ml were introduced. Twenty-four hours later they were returned to the cups. Spores grown in corn earworms were harvested 10-14 days after infection. Infected larvae were macerated in a Waring blender with 50-100 ml of glass distilled water (GDW). The resulting homogenate was filtered through plastic screening, pelleted twice at 1732xg, resuspended in 50-100 ml GDW and allowed to stand overnight at 4°C. Spores and light cell debris

settled last forming a light colored layer over the heavier fragments. This layer was removed with a Pasteur pipette in concentrations of up to 10^{10} spores/ml.

Indian meal moth and cabbage looper eggs were obtained from the Insect Attractants, Behavior and Basic Biology Lab (A.R.S., U.S.D.A.) at Gainesville, Florida. Indian meal moths were raised following the method of Silhacek and Miller (1972). Fifty-five mg of eggs (approximately 1800) were placed in plastic cups containing 5 g of medium. Five days after hatching, 1 ml of a suspension containing $5X10^6$ spores/ml of isolate 906 was added to 5 g of fresh medium, mixed thoroughly and placed in each cup. Twenty-four hours later the contents of each cup were transferred to 400 g of medium in 1 pint mason jars covered with filter paper. After 7-10 days the larvae were separated from the medium in a Berlese funnel (Rutter and Ferkovitch, 1973) and macerated in a tissue grinder. Spores were harvested as for corn earworms.

Cabbage loopers were reared according to Hensberry and Kinhaba (1966). Five-day-old larvae were starved for 24 hours and then allowed to feed on a small disc of medium on which had been deposited 1 ml of a suspension of Nosema trichoplusia containing 10^6 spores/ml. The larvae were allowed to feed for 24 hours and then were returned to the original medium. Spores were harvested 13-14 days later as from corn earworms.

Spore Purification

The spore suspensions were purified by a modification of the Ludox (silica colloid) density gradient method (Undeen and Alger, 1971). Two ml of suspension were overlayered onto 5 ml of 10% Ludox HS in 12 ml capacity cellulose nitrate centrifuge tubes and centrifuged at 30,000xg

for [illegible] minutes. The pellet of clean spores at the bottom of the tube was removed and washed twice by centrifugation at 1732xg for 10 minutes. Some samples contained small pieces of chitin which were removed by filtering through several thicknesses of Kimwipe . The final pellet was resuspended to a concentration of 10^{10} spores/ml in 0.5M $NaPO_4$ buffer, pH 7.5. The spores were disrupted in an MSK Braun Cell Homogenizer . Three ml aliquots of the buffered spore suspension containing $3X10^{10}$ spores, 6.5 ml of 0.5 mm diameter glass beads (Glasperlen or technical quality glass beads (Fetters Industries, Carlstadt, New Jersey)) and 2-3 drops of tributyl phosphate were added to each 75 ml disruption flask. The technical quality beads were cleaned in concentrated HCL prior to use (Neihof and Echols, 1968). The disruption flask was spun at 4000 rpm for 1.5 minutes in the cell homogenizer during which time it was cooled by passing CO_2 through the disruption chamber. Cell fragments were washed from the bead interstices by 4 successive washes of 0.5M $NaPO_4$ buffer, pH 7.5, and pelleted by centrifugation at 30,000xg for 20 minutes. The supernatant containing the hydrophilic protein was decanted and frozen for use in serology. The pellet was resuspended in 4:1:1 phenol-glacial acetic acid-GDW (w:v:v) (PAW) at a ratio of 1 ml PAW/10^{10} spores to extract the hydrophobic protein used in electrophoresis. After 2 hours at 4°C the suspension was centrifuged at 37,000xg for 1 hour and the supernatant decanted and stored at 4°C.

TABLE 1

SOURCE OF INOCULA

Microsporidan

Isolate #	Species	Host	Source	Origin
650	Nosema algerae	Anopheles albimanus	Hazard	Panama
906	Nosema plodiae	Plodia interpunctella	Lindegren	California
907	Nosema heterosporum	Plodia interpunctella	Lindegren	California
942	Nosema sp.[1]	Arzama densa	Hazard	Florida
954	Nosema sp.[1]	Acigrena ignitalis	Hazard	Florida
959	Nosema sp.	Ischnura ramburii	Hazard	Florida
960	Nosema sp.[1]	Nephelodes emmedonia	Brooks	N. Carolina
1051	Nosema sp.	Crayfish	Hazard	Florida
1074	Thelohenia sp.	Shrimp	Hazard	Florida
1116	Nosema bombycis	Bombyx mori	Wilson	Canada
1125	Nosema necatrix	Pseudaletia unipuncta	Maddox	Illinois

[1]Similar to N. necatrix.

CHAPTER II

SEROLOGY

Introduction

Serological techniques have been widely used to determine taxonomic relationships among parasitic Protozoa. The application of these techniques to the Microsporida has been hampered by the difficulty in producing sufficient quantities of spores and the lack of a suitable method for disrupting them. Recently, however, the work of Undeen and Maddox (1973) has expanded the number of microsporidian species available for mass rearing. Conner (1970) and Fowler and Reeves (1974a,b) have successfully employed the MSK Dreun Cell Homogenizer to disrupt spores and extract water soluble protein suitable for use as antigen.

Cali (1971) studied the development of Nosema bombycis N. apis and N. sunniculi with the electron microscope and suggested that the genus Nosema be restricted to those Microsporidans in which each sporont gives rise to two spores and in which the diplokaryon nuclear arrangement is present throughout the life cycle. The heterogeneous nature of this group has also been acknowledged in a taxonomic monograph by Sprague (in press). Possible candidates for a new genus are the group of Microsporidans similar to Nosema necatrix. The vegetative stages here are, like Nosema bombycis, equipped with diplokarya but when grown at low temperatures (below 26.7°C) mixtures of monospores (free single spores) and octospores (8 spores within a pansporoblast membrane) are produced. Since its original description as a dual infection of N. necatrix and

2 other similar organisms have been described from the Indian meal moth (Plodia interpunctella). Kellen and Lindegren (1969) described Thelohania nana as occurring only in dual infections of Plodia with Nosema plodiae and N. heterosporum and mention that at 27°C the Nosema sp. spores predominate. In addition, a number of Microsporidans morphologically similar to N. necatrix have been isolated at this and other labs from a variety of lepidopteran larvae from different geographic locations (Table 1).

Since the original isolate of this Microsporidan has been found to have a wide host range within the Lepidoptera (Madden, 1966) the taxonomic relationships between the various isolates are not clear. Kellen and Lindegren (1969) were able to infect 3 lepidopteran species with N. heterosporum but were less successful in transmitting N. plodiae to other hosts and concluded that the latter had a narrow host range. However, we have been able to infect corn earworms (Heliothis zea) with both of these Microsporidans which suggests that they may also have host ranges similar to N. necatrix.

This study is an attempt to clarify the taxonomic relationships within the N. necatrix group (including N. plodiae) and the relationship of the group itself to Nosema species meeting the criteria erected by Cali (1971) through the use of serological techniques. Microsporidans tested include N. bombycis, (The type species) N. algerae, N. plodiae, and 3 Nosemas similar to N. necatrix.

Materials and Methods

Serology

Hydrophilic protein extracts were concentrated to 1.2-2.4 mg/ml by vacuum dialysis through 5.5 mm diameter dialysis tubing. Dilute samples

were further concentrated by covering the tube with Carbowax (PEG 4000) for .5 to 1 hour. The protein content of the first extract of each microsporidian species was determined by the method of Lowry et al. (1951). The content of subsequent samples was estimated by the quantity of spores used. Rabbits were given 3 weekly intramuscular injections composed of 1 ml of antigen containing 1.2-2.4 mg of protein (approximately 3×10^{10} spores) with an equal volume of Freund's complete adjuvant. They were bled 3 days after the last injection. Serum immunoglobulins were precipitated by ammonium sulfate at 1/3 saturation (pH 7.8) and then dialyzed against sodium tetraborate buffered saline (pH 8.4).

Double immunodiffusion was accomplished in 2 mm thick 1.5% agar in .02 M $NaPO_4$ buffered saline (pH 7.5) on glass microscope slides. Wells 2.5 mm in diameter were cut using a Gelman immunodiffusion punch with the other wells 5 mm from the center well. Antigen concentrated to 2-5 mg/ml was placed in the outer wells and concentrated serum in the center. The plates were read after 48 hours, during which time the wells were refilled twice. The plates were washed, dried and stained in amidoschwarte (Uriel, 1964).

Results

Serology

Two basic types of precipitin bands were seen in antigen-antibody reactions involving anti-microsporidian serum. Strong reactions involving large quantities of immunoglobulin produced heavily stained areas which were designated as major bands in the text. Weaker bands containing relatively small amounts of reactants were designated minor bands.

Preliminary results using unconcentrated, unadsorbed antiserum to isolate 942, 960, Nuosma algerae, N. plodiae and CEN controls are seen in Figures 1-3. Nosema algerae, N. plodiae and isolate 942 showed reactions of non-identity with CEN. Isolate 960 which was an experimental attempt to clean spores by the triangulation method of Cole (1970), cross reacted with the host. Subsequent isolates were cleaned using Ludox density gradient centrifugation. Microsporidans 942 and 960 and N. plodiae showed considerable antigenic similarity while N. algerae showed reactions of non-identity with all three. N. plodiae developed 3 major bands, 942 1 major and 1 minor band, 960 one major band and N. algerae consistently showed 2-3 minor bands. CEN showed 1 major and 1 minor band. To produce readable plates using unconcentrated sera required refilling the wells 8-10 times over a period of 3 days. Since reactants this weak may not be strong enough to develop minor bands, subsequent work was done using concentrated antisera.

Controls

In order to detect any temperature related antigenic differences, antisera were produced against isolate 942 raised at 21°, 27° and 32°C. Results are shown in Figure 4 and indicate complete antigenic similarity between isolates raised at 27° and 32°C. When tested against the isolate raised at 21°C, however, a reaction of partial identity was observed with both (Figure 4).

To determine if aged spores differed antigenically, antiserum was prepared against spores of 942 which had been aged for 5 months. When compared to fresh spores raised at 21°, 27° and 32°C, no antigenic difference was noted with the spores raised at 27° or 32°C (Figure 5).

However, a slight reaction of partial identity was observed between aged spores and fresh spores raised at 21°C (Figure 5).

Figures 6-11 show the results of comparing the 9 antisera produced to that prepared against the CEW host. No cross reactions was observed in any of the major bands, however, slight reactions of identity were observed in all of the 942 antisera. To remove cross reacting gamma globulins, all antisera were adsorbed and tested against CEW antigen (Figures 12-13). No reactions with test antisera were observed and all subsequent plates were prepared using adsorbed antisera.

Test Reactions

Anti-N. algerae Serum

This serum gave 4-5 precipitin bands with its homologous antigen (Figure 17). The darkest band formed slightly closer to the serum well toward which it curved. All other bands were much more indistinct. One or 2 bands formed between the major band and the serum well, and an additional 3-4 between the major band and the antigen well. Two of these were located near the major band and another pair (Figures 14, 16-17), which sometimes appeared as a single line (Figure 15) formed near the antigen well. All of the bands between the major band and the antigen well curved toward the antigen well. None of the bands formed to anti-N. algerae serum showed reactions of identity with any of the other sera tested (Figures 14-17).

Anti-N. bombycis Serum

Against its homologous antigen anti-N. bombycis serum produced 7 bands oriented in 3 groups (Figure 22). The group nearest the serum well contained the major band which curved toward the well and a minor

band which followed the contour of the first. Midway between the two wells was a group of 3 closely spaced minor bands with the 2 outer being darker than the one in the middle. Two minor bands were located near the antigen well and were strongly curved towards it. In reactions comparing anti-N. bembycis serum to other antisera, some of these bands were suppressed and usually only the middle group of 3 was clearly expressed (Figures 17-19). Anti-N. bembycis serum did not cross react with any of the other antigens (Figures 17-19).

Anti-N. plodiae Serum

Reactions involving anti-N. plodiae serum and its homologous antigen typically produced 2 major bands, one midway between the wells and the other closer to the serum well (Figure 15). Some plates developed a minor band between the major ones (Figures 15-17) and often the darker major band separated into several bands (Figure 16). A minor band was usually present between the antigen well and the darkest major band. Anti-N. plodiae serum cross reacted with that prepared against isolates 942 and 960 (Figures 14, 15).

The inner major band of N. plodiae developed a reaction of partial identity with the major band of isolate 942, with the spur indicating a unique antigen in the N. plodiae band (Figure 14). In addition, one of the minor bands of N. plodiae ends in a sharp curve indicating possible antigenic similarity (Figure 14). The inner major band of N. plodiae forms a double spur reaction with the major band of isolate 960 (Figure 15).

Anti-Nosema sp. (942) Serum

Reactions involving anti-942 serum and its homologous antigen produce a single major band midway between the wells and 2 lighter bands closer to the serum well (Figures 4-5). In reactions involving other antisera, additional bands appeared between the major band and the antigen well (Figure 14). Anti-942 serum did not cross react with antigens from N. kambycie (Figure 18), N. algerae (Figure 14), or Nosema sp. 954 (Figure 20) but reactions of partial identity were seen against N. plodiae (Figure 14) and Nosema sp. 960 (Figure 20). Isolates 942 and 960 share at least 2 common antigens (Figure 20). These included the major bands of both isolates plus 1 minor band (Figure 20). The minor band closest to the serum well showed a reaction of non-identity with isolate 960 (Figure 20) and may be considered specific to isolate 942. The major band of isolate 942 cross reacted with the major band of N. plodiae nearest the serum well (Figure 14). In addition, the minor band of N. plodiae nearest the antigen well curved sharply as it approached the isolate 942 antigen well indicating that it is a common antigen (Figure 14). Thus, isolate 942 and N. plodiae had at least 2 common antigens.

Anti-Nosema sp. (960) Serum

Reactions between anti-960 serum and its homologous antigen resulted in one major band midway between the wells which curved slightly toward the serum well and two minor bands between the major line and the antigen well which curved toward the antigen well (Figure 24). In reactions involving pooled antisera this reaction tended to become masked and difficult to read and presumably isolate 960 produced the weakest of the 6 antisera. Anti-960 serum did not cross react with antigens of N.

bombycis (Figure 18), N. algerae (Figure 15), and Nosema sp. 954 (Figure 20), but did develop lines of partial identity with Nosema sp. 942 (Figure 20) and N. plodiae (Figure 15). The major band of isolate 960 showed a reaction of identity with the major band of Nosema sp. 942 as did the minor band nearest the antigen well (Figure 20). Isolate 960 is thus antigenically similar to 942 except for the minor line near the serum well which is present in 942 but absent from 960. In reactions against N. plodiae, isolate 960 was similar to 942 in that the major band of 960 cross reacted with the inner major band of N. plodiae and the outer minor band of N. plodiae curved sharply toward the serum well (Figure 15). Unlike the reaction between 942 and N. plodiae, however, the major band reaction between 960 and N. plodiae formed a double spur which indicated that the two isolates contained at least one common and one unique antigen.

Anti-Nosema sp. 954 Serum

This serum gave 5 bands when reacted with its homologous antigen (Figure 23). Four of these bands were located midway between the wells and were of similar strength. The 2 outer bands curved slightly toward the antigen well while the inner pair curved toward the serum well. The fifth band was very faint and was located near the antigen well toward which it showed a pronounced curve. Anti-954 serum did not cross react with any of the other antigens (Figures 16, 19-20).

Discussion

Serology

The results of this study indicate antigenic similarity between N. plodiae and isolates 942 and 960. Nosema algerae, N. bombycis and Nosema sp. 954 were not antigenically related to each other or to the

three cross reacting species: Nosema spp. 942 and 960 have at least 4 common antigens as do Nosema sp. 942 and N. plodiae. Nosema sp. 960 and N. plodiae have 1 common antigen. Nosema plodiae also has a number of unique antigens indicating that isolates 942 and 960 are more closely related to each other than either is to N. plodiae. Nosema sp. 942 is more closely related to N. plodiae than is Nosema sp. 960 as it has 1 more common antigen.

Fowler and Reeves (1974b) subjected water soluble protein from 2 spp. of Microsporida to electrophoretic analysis (Davis, 1964). Their results showed considerable differences in protein spectra of Microsporidans raised at different temperatures and after prolonged storage (30 days). In the present study different temperatures caused a slight antigenic difference but storage of as much as 5 months caused no effect. Fowler and Reeves (1974b) attribute storage effects to the breakdown of high molecular weight precursors during spore maturation. If such cleavage did not involve antigenic sites it would be more readily detected by electrophoresis than by immunodiffusion techniques. Nosema sp. 942 was chosen for monitoring temperature changes because it grew more readily in available host insects than other isolates. Unfortunately this a N. necatrix like organism and small amounts of octospore are produced at 21°C. Accurate records were not kept on octospore/monospore proportions but Fowler and Reeves (1974c) reported a ratio of 8.7 monospores to 1 octospore for N. necatrix recovered from CSW hosts reared at 21°C. If octospores differ antigenically from monospores this could account for the reaction of partial identity seen in Figures 4-5. Fowler and Reeves (1974a) found that at 15.6°C octospores predominate in a ratio of 1/0.3. Presumably antiserum against such a mixture should

Five a similar but much stronger reaction that that seen in Figures 4-5. Unfortunately, isolate 942 developed in insufficient quantity at this temperature for serological analysis. The effect of temperature on spore antigens should be investigated using a non-polymorphic species such as M. barbycis or N. trichoplusia. Fowler and Reeves (1974b) found that N. trichoplusia flac electrophoresis spectra showed less temperature related variation than did those of N. necatrix, so some of the N. necatrix variation may have been due to the polymorphic spores.

Johnson and Powell (1974) reported that the electrophoretically distinguishable alcohol dehydrogenases of Drosophila melanogaster could be correlated with heat and cold shock. The variation in hydrophilic protein seen in the present study and reported by Fowler and Reeves (1974b) may be related to temperature related isozymal differences. Fowler (1971) ran spectrophotometric assays for the presence of malate and lactate dehydrogenases, and alkaline phosphatases in disrupted spores. He also attempted to detect esterase and peroxidase activity through disc electrophoresis and malate dehydrogenase activity in intact spores. All assays were negative but more work in this area is needed before temperature related isozymes can be ruled out.

Fowler and Reeves (1974b) found that hydrophilic protein spectra varied when isolates were raised in 2 different hosts. Both the triangulation method employed by Fowler and Reeves (1974b) and the density gradient method of Undeen and Algar (1971) used in this study produce spore suspensions with a high degree of purity. Therefore, the observations of Fowler and Reeves (1974b) and the assay reactions with heat antigen seen with anti-Nosema sp. 942 sera may have been caused by host protein adhering to, or contained within the spore wall. This could

be determined by adsorbing homologous spore isolates with anti-host serum bound to an affinity gel. If then electrophoretic spectra were constant for the same species raised in different hosts, some host material is incorporated into the spores. Continued spectrum variation after this treatment would indicate changes in the protein complement of the parasite induced by different hosts.

Where applicable the results of this study corroborate the findings of Fowler and Reeves (1974a). These authors were unable to differentiate between N. plodiae, N. necatrix, and 4 other microsporidian isolates using disc electrophoresis. The other isolates described as Thelohania sp., Nosema sp. or Nosema-Thelohania may have been conspecific with N. necatrix. Nosema sphingidis was originally isolated from the tobacco hornworm (Manduca sexta) by Brooks (1970). Its spore dimensions (3.8-5.4X 1.9-2.7u) overlap with those of N necatrix (5.06-6.05X 2.32-2.82) (Kramer, 1965), and it has a wide host range. Brooks (1970) did not report finding osteospores but his material was raised at 27°C so none could be expected. Nosema sphingidis, then, may also be a strain of N. necatrix. In the present study 2 N. necatrix-like species were found to cross react with N. plodiae but not with other Nosema sp. Since it was possible to differentiate serologically the 3 cross reacting species, serology may be considered more sensitive than disc electrophoresis in separating microsporidian isolates.

Due to its sensitivity and to the fact that it is not affected by storage of spores up to 5 months before disruption, serology is a good method for separating morphologically similar microsporidian isolates. Rearing temperature may have some effect but it is slight when compared to that resulting from isolate differences (Figures 4-5) and, as stated

above, the cross reaction seen may have been due to spore polymorphism. Future work then should be directed toward resolving the question of temperature and host effects on microsporidian antigens.

The major disadvantage to serology is the large quantity of spores required. Immunodiffusion techniques require at least 6×10^{10} spores to produce usable titer in rabbits plus an additional 10^{10} or more to set up gel diffusion tests. This quantity of spores can be produced in some of the Nosema spp. infecting Lepidoptera and Orthoptera but most other Microsporidans are available in much smaller quantities only. Injection of Ludox cleaned spores into the host hemocoel has been found to widen the host range of N. algerae (Undeen and Maddox, 1973) as does starving the host before feeding a concentrated spore suspension. If these techniques can be applied to other Microsporidans the taxonomic value of serology will be enhanced.

Figures 1-3. Reactions using unconcentrated, unadsorbed antisera
Pooled antisera in center well ca, corn earworm
host, el, N. elgarae, pl, N. plodiae, 942,
Nosema sp. 942, 960, Nosema sp. 960.

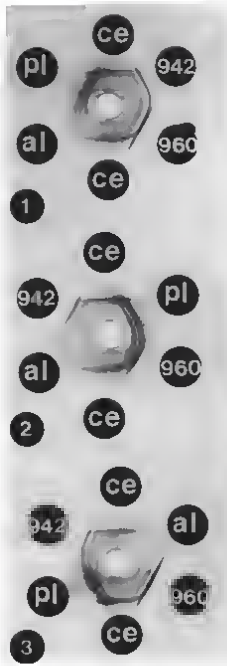

ce
pl
942
al
960
ce
1
ce
942
pl
al
960
2
ce
ce
942
al
pl
960
ce
3

Figure 4. Reactions involving isolates of Nosema sp. 942 raised at different temperatures. Pooled antisera in center well. 21, isolate 942 raised at 21°C, 27, isolate 942 raised at 27°C; 32, isolate 942 raised at 32°C.

Figure 5. Reactions involving fresh isolates of Nosema sp 942 with isolates from spores aged for 5 months. Pooled antisera in center well. A, isolate 942 raised at 27°C and stored 5 months; 21, isolate 942 raised at 21°C, 27, isolate 942 raised at 27°C, 32, isolate 942 raised at 32°C.

Figure 6. Control reactions against corn earworm host using concentrated, unadsorbed antisera. Pooled antisera in center well. ce, corn earworm; 21, isolate 942 raised at 21°C, 27, isolate 942 raised at 27°C.


21
21
32
21
4
21
32
A
A
27
5
27
ce
21
6

Figure 7. Control reaction against corn earworm host using concentrated unadsorbed antisera. Pooled antisera in center well. ce, corn earworm, A, aged spores of isolate 942, 32, isolate 542 raised at 32°C.

Figure 8. Control reactions against corn earworm host using concentrated, unadsorbed antisera. Pooled antisera in center well. ce, corn earworm, al, N. algerae.

Figure 9. Control reactions against corn earworm host using concentrated, unadsorbed antisera. Pooled antisera in center well. ce, corn earworm; 954, Nosema sp. 954, 960, Nosema sp. 960.

A
ce
32
7

al
ce
8

954
ce
960
9

Figure 10. Control reaction against corn earworm host using concentrated, unadsorbed antiserum. Pooled antisera in center well. ce, corn earworm, bo, N. bombycis.

Figure 11. Control reaction against corn earworm host using concentrated, unadsorbed antiserum. Pooled antisera in center well. ce, corn earworm, pl, N. plodiae.

Figure 12. Control reactions against corn earworm host using concentrated, adsorbed antisera. Corn earworm antigen in center well. ce, corn earworm, 954, Nosema sp. 954; 960, Nosema sp. 960; bo, N. bombycis, al, N. algerae; pl, N. plodiae.

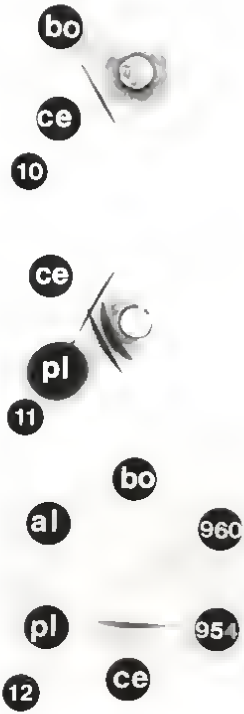
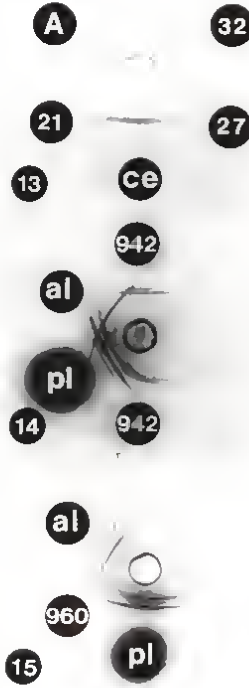

bo
ce
10
ce
pl
11
bo
al
960
pl
954
12
ce

Figure 13. Central reactions against corn earworm host using concentrated, adsorbed antisera. Corn earworm antigen in center well. cw, corn earworm; 21, isolate 942 raised at 21°C; 27, isolate 942 raised at 27°C, 32, isolate 942 raised at 32°C, A, aged spores of isolate 942.

Figure 14. Test reaction using concentrated, adsorbed antisera. Pooled antisera in center well. al, N. algerae; pl, N. plodiae, 942, Nosema sp. 942.

Figure 15. Test reaction using concentrated, adsorbed antisera. Pooled antisera in center well. al, N. algerae; pl, N. plodiae; 960, Nosema sp. 960.


A
32
21
27
13
ce
942
al
pl
14
942
al
960
pl
15

Figures 16-18. Test reactions using concentrated, adsorbed antisera. Pooled antisera in center well. al, *N. algarae*, pl, *N. pledinae*, bo, *N. bombycis*, 942, *Nosema sp.* 942; 954, *Nosema sp.* 954, 960, *Nosema sp.* 960.


al
954
pl
16
pl
bo
al
17
942
bo
960
18

Figures 19-20. Test results using concentrated, adsorbed antisera. Foaled antisera in center well, bc, *N. bombycis*, 942, *Nosema* sp. 942; 954, *Nosema* sp. 954, 960, *Nosema* sp. 960.

Figure 21. Diagrammatic representation of Figure 20.


bo
954
19
942
954
960
20
942
942
954
960
942
21

Figure 22. Reaction of anti-*M. bombycis* serum and its homologous antigen. Antiserum in center well.

Figure 23. Reaction of anti-*Nosema* sp. 954 serum and its homologous antigen. Antiserum in center well.

Figure 24. Reaction of anti-*Nosema* sp. 960 serum and its homologous antigen. Antiserum in center well.


bo
22
23
954
960
24

CHAPTER III

MICROELECTROPHORESIS

Introduction

Recently there has been a dramatic increase in the number of morphologically similar species of the microsporidian genus Nosema described from the Lepidoptera. According to Nordin and Maddox (1974) there are approximately 55 species of Nosema described from this order, however, many of these are synonymous (Weiser, 1961) or inadequately described, making the naming of the new species difficult. The 2 polyacrylamide electrophoresis methods used by Fowler and Reeves (1974a,b) may be of considerable taxonomic value here. Of the two methods the low pH system would seem the better choice as the spectra are not affected by differing temperatures, hosts or age of spores. Furthermore, extraction with phenol-glacial acetic acid-water (PAW) yields far more protein than does extraction with sodium phosphate buffer (Fowler and Reeves, 1974a,b). Unfortunately, the method still requires 10^9 or more spores, a quantity which may be difficult to obtain from small or lightly infected larvae. Recognising this limitation, Fowler and Reeves (1974a) suggested adaptation of the low pH (hydrophobic extract) system to micro methods such as used by Friz et al. (1970), who analysed protein extracted from individual amoebae.

Microelectrophoresis, which employs 0.2-1.0 mm i.d. capillary tubes instead of the standard 5 mm diameter size, was first reported by Greenbaum (1965) who used it to study chironomid salivary gland secretions.

Hayden et al. (1967) and Hayden and Lange (1968) used similar methods on individual nerve cells and were able to detect protein in nanogram quantities. Thus, microelectrophoresis would seem to be an excellent technique for investigating taxonomic relationships in the Microsporida. The work presented below is an attempt to adapt the methods developed by Fowler and Reeves (1974a) for analysis of phenolic protein extracts of disrupted microsporidian spores to micro methods.

Materials and Methods

Microelectrophoresis

Microelectrophoresis was carried out in 1.1-1.3 mm I.D. hematocrit capillary tubes, in 1.7 mm I.D. glass tubing and in standard 5 mm diameter tubes. All tubes were 75 mm long. The stock gel solution contained 10 g acrylamide, 0.26 g N, N^1 methylenebisacrylamide, 20 g urea, 46.6 ml glacial acetic acid, and GDW to a total volume of 100 ml (Razin and Hattam, 1967). To prepare the gels, 0.012 ml of N, N, N^1-tetramethylenediamine was added to 1.6 ml of the stock acrylamide solution. The catalyst was 0.4 ml of 1.2% ammonium persulfate to which had been added 0.074 g of urea giving a final concentration of 7.5% acrylamide with 0.0195% bis. Fifty ul of acrylamide was added to each 1.1-1.3 mm I.D. tube via a 100 ul Hamilton syringe after one end of the tube had been sealed with hematocrit clay. The larger tubes were sealed with Parafilm and enough acrylamide added to form a gel 50 mm long. After overlayering the gel with 10% acetic acid, polymerization occurred in a 50°C water bath for 30 minutes. Tubes not used immediately were stored at 4°C. After polymerization the 10% acetic acid was drawn off and the PAW protein extract added and overlayered with 10% acetic acid. The

smallest tubes received 4-8 ul of protein extract, the 1.7 mm I.D. tubes 10-30 ul, and the 5 mm I.D. tubes 100 ul. Electrophoresis was conducted at pH 2.2 with 10% acetic acid used as electrolyte in a standard Buchler electrophoresis apparatus at room temperature. At this pH proteins migrate toward the cathode which was placed in the lower chamber. The 1.1-1.3 mm I.D. tubes received a constant current of 0.3 mA, the 1.7 mm I.D. tubes 0.75 mA, and the 5 mm I.D. tubes 2 mA throughout the run. Electrophoresis lasted 70-90 minutes. After electrophoresis gels were removed from the capillary tubes and fixed, they were stained in 1.5% amidoschwartz in 7.5% acetic acid. The 1.1-1.3 I.D. size was stained for 30 minutes and the larger tubes 60 minutes. Gels were destained and stores in aqueous solutions containing 10% each of methanol and acetic acid.

Results

Microelectrophoresis

Results obtained for N. plodiae, N. heterosporum and Nosema sp. 942 are presented in diagram form in Figure 25. Major bands are represented by solid lines, minor by stippling and the inulin marker by diagonal lines. A total of 22 different bands was seen in the 3 spectra and are represented by arabic numerals. Bands 1-7, 9, 12-15, 17-18, and 20-22 were considered to have no homologues in spectra of other isolates while band 10 was shared by all 3. Nosema sp. 942 shared homologous bands 8, 16, and 19 with N. heterosporum and band 11 with N. plodiae. N. heterosporum and N. plodiae shared only band 10. Nosema sp. 942 had the fewest bands with 8, N. plodiae had 9 and N. heterosporum 11.

In order to quantify the taxonomic relationship between the 3 species tested, the coefficient of similarity was determined as by Whitney et al. (1968) by the following formula:

$$Cs = \frac{\text{Number of homologous bands}}{\text{Number of different bands + number of homologous bands}} \times 100$$

The results of these calculations are seen in Table 2.

TABLE 2

COEFFICIENTS OF SIMILARITY (Cs) VALUES

Isolate	Nocsma sp. 942	N. plodise	N. heterosporum
Nocsma sp. 942	100	13	26
N. plodise	13	100	5
N. heterosporum	26	5	100

Discussion

Microelectrophoresis

Although Fowler (personal communication, 1974) was able to use the low pH system routinely in standard sized tubes, considerable difficulties were encountered in the present study in both large and micro tubes. Resolution was consistently poor, and the bands appeared faint and diffuse with considerable background staining. Additional difficulties were encountered in removing gels from the tubes. Gels 1.7 mm in diameter could generally be removed intact by carefully forcing a fine wire between gel and tube while forcing water into the tube from a syringe. If not previously loosened with the wire, the gels stretched when reamed with water causing corresponding distortion of the protein

spectra. The 1 cm diameter gels were the most difficult in this regard. Friz et al. (1970) and Hayden and Langa (1968) forced the gels out with a tight fitting wire. Grossbach (1965) placed the tubes in dry ice then forced out the frozen gels with water from a syringe. At best these methods freed approximately 75% of the gels intact and often 100% of a given run was macerated. Recovery rates were not improved by loosening the gel with a fine wire and the wire had the added disadvantage of scratching the gel. All attempts to smash the capillary severely damaged the contents.

The most likely cause of these difficulties is incompletely polymerized gels. Raxin and Rettes (1967) carried out polymerization at 37°C for 45 minutes. Gels used in the present study did not polymerize at this temperature and at 50°C Fowler (1971) required from 20 minutes to several hours. Furthermore, the methods of these authors differ with regards to percentage of catalyst and sequence of mixing the components of the gel. In light of these discrepancies possibly some change in the formula of the gels (i. e. an increase in the percentage of catalyst) would produce more uniform results.

Insulin performs adequately as a marker protein in this system especially when used at a concentration of 0.5 mg/ml of PAW. Lower concentrations are sometimes difficult to distinguish from microsperidium bands or background staining. As can be seen from Figure 25, however, many microsperidian proteins migrate faster than insulin and there is the possibility of interference. This situation could be circumvented by running control tubes without insulin but this would partially negate the advantage of using a marker over the more cumbersome method of Johnson et al. (1967). An alternative would be the use of a more rapidly

migrating marker. Possible candidates may be found among the histones which are low molecular weight basic proteins that should migrate rapidly in this system.

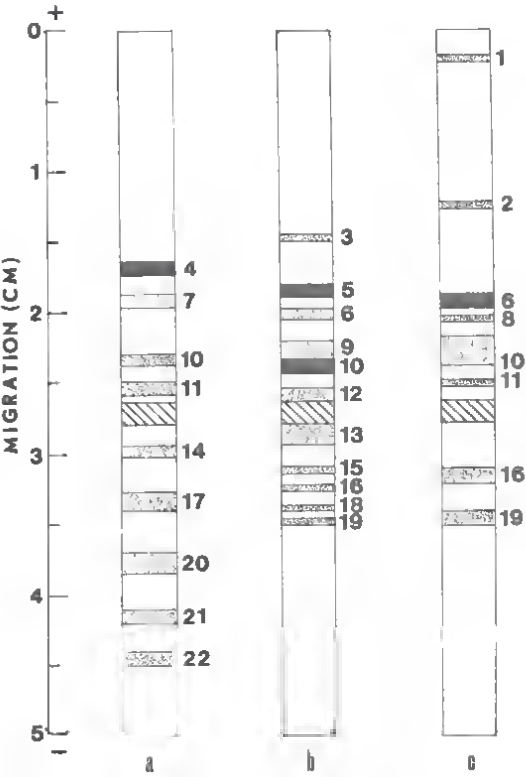
To minimize artifacts caused by polymerization difficulties, the protein spectra diagrammed in Figure 25 were replicated several times with good agreement among successive runs. The 3 Microsporidans tested are readily distinguishable on the basis of their protein spectra. Nosema sp. 942 and N. heterosperum have 4 homologous proteins and thus may be more closely related to each other than either is to N. plodiae, which shares 2 proteins with each. Fowler and Reeves (1974a) found that the spectrum of N. plodiae was identical to that of N. necatrix and 5 other organisms variously described as Nosema sp. or Nosema-Thelohania. All of these may have been N. necatrix as this is a widespread and commonly encountered lepidopteran pathogen. The spectra obtained in the present study for N. plodiae and Nosema sp. 942, which is morphologically similar to N. necatrix, do not correspond to those reported by Fowler and Reeves (1974a). This discrepancy is probably in part due to idiosyncrasies of the individual technicians, but it may also be the result of strain difference or contamination.

The coefficient of similarity (Cs) values obtained in this study were considerably lower than those reported by Fowler and Reeves (1974a). These authors reported N. plodiae and N. necatrix as having a Cs of 100, while in the present study N. plodiae and Nosema sp. 942 had a Cs of 26. Furthermore, Fowler and Reeves (1974a) found a Cs of 42-56 between Thelohania legeri and the various species of Nosema they tested. This value theoretically should have been much smaller that that obtained for 2 species in the same genus.

Such results are most easily explained through differences due to polymerization of gels used in the present study. The 3 Microsporidans used are probably closely related as Nosema sp. 942 produces octospores and N. algerae and N. heterosporum are accompanied by similar forms originally described as Thelohania nana (Kellen and Lindegren, 1969). In light of this morphological similarity much higher Cs values should have been obtained.

In spite of the dubious results obtained so far, microelectrophoresis has great potential value in the field of microsporidian taxonomy as spores are frequently difficult to obtain in sufficient quantity. The 1 mm diameter tubes used 4 ul of PAW extract at the concentration used by Fowler and Reeves (1974a), a reduction in volume of 25% and the equivalent of $1.3X10^7$ spores. CSN larvae may contain as many as $8X10^9$ spores, however larval Anopheles quadrimaculatus contain on more than $1.5X10^6$ spores. Hopefully further work on this technique will result in greater reliability.

Figure 25. Diagrammatic representation of 1.7 mm diameter micro-electropheresis gels. Individual numbers adjacent to bands indicate homologous bands. (a) Nosema pieckae. (g) Nosema heteroperum. (o) Nosema sp. 942.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MIGRATION (CM)
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12
13
15
16
18
19
1
2
6
8
10
11
16
19
a
b
c

CHAPTER IV

SPORE COAT ELECTROPHORESIS

Introduction

As the number of described species of Microsporida increases it becomes increasingly evident that species separation based solely on morphological characters is inadequate. Such characters as spore shape and size have been shown to be variable (Kudo, 1924; Blunck, 1954; Walters, 1958; Kramer, 1960) and often there is much overlapping between species (Nordin and Maddox, 1974). Other characters such as polar filament length, host specificity, tissue specificity are also of dubious value (Fowler, 1971). The separation of species by electrophoretic analysis developed by Fowler and Reeves (1974a,b) is an important development in the field of microsporidian taxonomy. Unfortunately, pursuit of this type of work depends on the availability of the MSK Braun Cell Homogenizer , a relatively scarce and expensive piece of equipment. In order to circumvent this difficulty the present work was aimed toward utilization of the outer proteinaceous spore coat for electrophoretic analysis.

As early as 1921, Kudo discovered the probable presence of chitin in the microsporidian spore coat (Sprague, 1970). This observation was confirmed by the histochemical work of Dissanaike and Canning (1957) and Vavra (1966). More important to the present study, Vavra (1966) subjected alkaline hydrolyzed spore shells to chromatographic analysis and reported the presence of 11 different amino acids. He concluded that the pore

shell contained protein. Furthermore, his observations of spores of Gaudospora showed that during alkaline hydrolysis only the outer portion of the spore coat is dissolved. Vavra (1967) subjected spores of Nosema bombycis, N. meamilli, and Pleistophora schafernai to several proteolytic enzymes and found them to be resistant. They were also resistant to the action of chitinase until the outer coat was removed by alkaline hydrolysis, after which they were dissolved. Vavra (1968) studied the development of Gaudospora simulii with the electron microscope and found that the spore coat is composed of an outer electron dense "exospore" which is affected by alkaline hydrolysis and an inner electron transparent "endospore" which is resistant. Thus Vavra (1968) concluded that this outer "exospore" contains protein. The electron microscopic work of many authors has shown that this electron dense layer is characteristic of all microsporidian spores and should then be a suitable target for electrophoretic analysis if solubilized.

Bagdasarian et al (1964) employing a mixture of phenol, acetic acid and water (PAW) extracted up to 95% of the total nitrogen content of a leaf and found that the protein extracted by this method, although probably denatured was not hydrolysed. Subsequently, Hamin and Rottma (1967) adapted the polyacrylamide gel electrophoretic methods of Davis (1964) to utilise phenol-acetic acid-water (PAW) extracts of membrane bound protein of Mycoplasma organisms. Fowler and Reeves (1974a) used PAW extracts of disrupted microsporidian spores to determine taxonomic relationships and found that the spectra were stable with regard to differing rearing temperatures, hosts and spore ages. Thus PAW was chosen for use in the present study.

Materials and Methods

Spore Coat Electrophoresis

Polyacrylamide gels were prepared as for micro-disc electrophoresis (page 35). Ludox cleaned spores were pelleted at 1732xg for 10 minutes and resuspended for 2 hours in PAW containing .5 mg of insulin/ml One ml of PAW was used for each 10^{10} spores. After extraction the spores were removed by centrifugation at 1735xg for 15 minutes and the supernatant decanted. The supernatant was analyzed electrophoretically as described for micro-electrophoresis (page 35).

Results

Spore Coat Electrophoresis

Diagrammatic representations of the 5 spore coat protein spectra are seen in Figure 26. All microsporidian bands were faint and are stippled in the diagram. The diagonal lines denote the insulin marker. The 5 microsporidian spp. tested showed a total of 8 different protein bands numbered in the diagram from the anode. Isolate 1074 (Thelohania duorarum) produced the fewest bands with 1, isolates 1051 and 959, 2, isolate 960, 3 and Nosema sp. 942, 4. All Microsporidans shared band 7 but all other bands were unique for each species.

Figure 27 represents isolate 942 treated as above except the spores were frozen several weeks before extraction with PAW. The spectrum contains 9 bands, 2 of which correspond to bands 2 and 7 in the nonfrozen spore coat preparation (numbers on left side of diagram). There is a high degree of similarity between this spectrum and that derived from disrupted spores. Six of the 9 bands correspond to those seen in Figure 25 (numbers on right side of diagram).

Discussion

Spore Coat Electrophoresis

Electrophoretic analysis of PAW extracts of spore coat protein appears to be an excellent method of separation of species of Microsporida. The procedure is relatively rapid as the cumbersome step of disruption of spores is eliminated. This in turn eliminates the need for the MSK Braun Cell Homogenizer, a relatively scarce piece of equipment, and also time consuming ultracentrifuge runs. The method is sensitive enough to separate morphologically similar species as can be seen by the spectra for isolates 942 and 960 (Figure 26), but comparisons with other techniques such as electrophoresis of PAW extracts of disrupted spores, serology and isoelectric focusing will require more work using similar microsporidian isolates.

A disadvantage of the spore coat technique is the extreme weakness of the resulting bands. Possibly this could be overcome by using a larger number of spores per unit volume of PAW. Little work was done in this area in the present study as one of the original objectives was to correlate spore coat bands with those obtained from disrupted spores. A few isolates were done using 5×10^7 spores of isolate 942/0.1 ml of PAW but the resultant background staining offset any advantage gained by concentrating the protein in the bands. However, Fowler and Reeves (1974a) reported variation in background staining in different microsporidian isolates so this line of investigation should be pursued further. Bagdasarian et al. (1964) were able to extract up to 95% of the total protein content from their experimental material indicating that PAW is extremely efficient. Other materials such as mercaptoethanol and dithiothreitol may be used to suspend membrane bound protein which can be

then electrophoresed in the presence of sodium dodecyl sulfate (Shapiro et al., 1967; Weber and Osborn, 1969). This method can also be used to determine the molecular weights of the proteins involved and allows the use of bromophenol blue marker dye. Possibly then, SDS-polyacrylamide electrophoresis will prove to be of value in analysis of spore coat proteins.

Although species may be readily distinguished by this method, the relative paucity of bands makes taxonomic relationships difficult to determine. Band 7 which is common to all isolates also corresponds to band 11 and possibly 12 in Figure 25 and may be considered as having come from the spore coat in the disrupted material. The fact that none of the other spore coat bands in isolate 942 correspond to the disrupted preparations may be due to the very faint spore coat bands being masked by background staining.

Because of the close similarity of Figure 27 to the spectrum of isolate 942 seen in Figure 25, it seems likely that freezing spores of this isolate causes some disruption. Microsporidian spores vary in their response to low temperature. Vavra and Undeen (1970) found that spores of N. algerae lost all infectivity when frozen. Henry (1971) routinely stored spores of N. locustae at -4°C apparently with little loss of activity. Moffat and Wilson (1971) froze spores of N. apis for short periods of time at -20°C with few adverse effects, a phenomenon also noticed by Bailey (1972) who successfully lyophilized N. apis and found infectivity up to 2 years later. Possibly, though, in all these cases some spores had burst. Alternatively, freezing may have rendered the spore coat more susceptible to the action of PAN. In this case most

of the bands seen in disrupted preparations may be derived from the spore coat and not from internal membranes.

Figure 26. Diagrammatic representation of spore coat disc gels. Identical numbers adjacent to bands indicate homologous bands. (a) Nosema sp. 942. (b) Nosema sp. 959. (c) Nosema sp. 960. (d) Microsporidan 1051. (e) Thelohania sp. 1074.

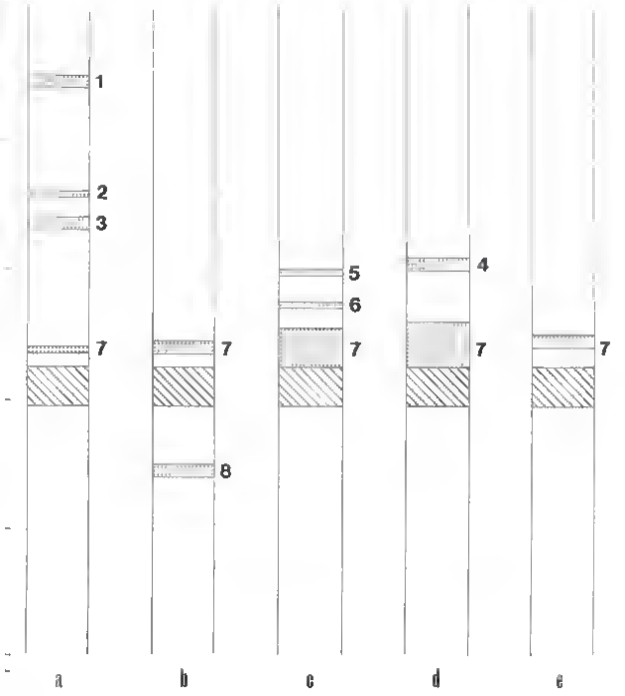
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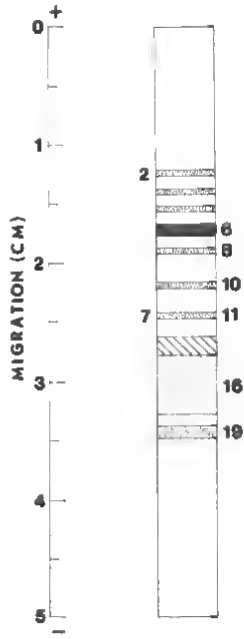
Figure 27. Diagrammatic representation of spore coat disc gel prepared from frozen spores of Nocardia sp. 942. Numerals at left correspond to those in Figure 26. Numerals at right correspond to those in Figure 25.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MIGRATION (CM)
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CHAPTER V

ISOELECTRIC FOCUSING

Introduction

Isoelectric focusing, whether used in a preparative or in an analytical technique, has been found to be one of the most sensitive methods known for the separation of proteins and other amphoteric molecules. Much early work was done using sucrose density gradient columns in which the emphasis was on preparation. However, in 1968 several workers simultaneously described gel-rod techniques that were much more suited to analysis of protein spectra (Riley and Coleman, 1968; Fawcett, 1968). In addition, Leaback and Rutter (1968) and Awdeh et al. (1968) reported the use of thin layers of acrylamide. Vesterberg (1972) modified the slab technique to include efficient cooling that allowed a reduction of the time required for focusing (Soderholm et al., 1972). At present the technique is sensitive enough to separate proteins whose isoelectric points differ by as little as 0.005 pH units (Righetti and Drysdale, 1974). A number of authors have reported obtaining better resolution with isoelectric focusing than with disc electrophoresis (Peterson, 1971, Drewett and Greg, 1972, Jeppsson and Berglund, 1972; Rowley et al., 1972).

At present only Fowler and Reeves (1974a,b) have attempted to separate species of Microsporida on a biochemical basis. They were successful in separating to the species level using both conventional electrophoretic methods (Davis, 1964) and the low pH modification of

Baxin and Rottem (1967) which uses a mixture of phenol acetic acid and ester to solubilize membrane bound protein. Both methods showed limitations. Protein spectra resulting from the method of Davis (1964) showed considerably variation when differing aged spores of the same species were analysed. Smaller differences occurred when spores were raised in different hosts or in the same hosts at different temperatures. Although different species also showed different spectra the amount of spurious variation is enough to cast suspicion on this technique. The spectra gained through the use of phenol-acetic acid-water extracts were much more stable showing variation only among differing species but Fowler and Reeves (1974a) reported a complex of six microsporidian isolates which included 3 distinct, described species which could not be distinguished. The following study was undertaken in order to determine the suitability of isoelectric focusing for separation of species of Microsporida.

Materials and Methods

Isoelectric Focusing

Isoelectric focusing was done on 1 mm thick polyacrylamide slabs measuring 90 X 240 mm obtained from LKB (Ampholine PAGplates). The pH range was 3.5-9.5, the polyacrylamide concentration 5% with 3% cross linkage. Power and cooling was supplied by an LKB Multiphor apparatus. Hydrophilic protein extracts were concentrated to 4-5 mg/ml, 15 ul of which was absorbed onto 5x10 mm pieces of filter paper placed on the cathode edge of the PAGplate. Anode electrolyte was 1M H_3PO_4 while that of the cathode was 1M NaOH. Runs were made at 5°C with the current set at 1 mA/sample and returned to this figure at 10-minute intervals until

until 750 volts was reached. This normally took 2.5 hours after which focusing was continued at 750 volts for an additional hour. The plates were fixed for .5-1 hour in a solution containing 150 ml methanol, 350 ml GDW, 17.25 g sulfosalicylic acid and 57.5 g trichloracetic acid. The protein was stained for 10 minutes at 60°C in 100 ml of ethanol-acetic acid-GDW (25:8:62) containing .115 g of Coomassie Brilliant Blue R250. Excess stain was removed in several washes of ethanol-acetic acid-GDW. The plates were then transferred to a preservative solution composed of 50 ml glycerol in 500 ml of ethanol-acetic acid-GDW for 1 hour, dried overnight and covered with a sheet of clear plastic.

Results

Isoelectric Focusing

The 7 microsporidian isolates tested yielded from 16 to 28 bands when subjected to isoelectric focusing. The majority of these bands focused between pH 5-8 (Figure 28).

Controls

Control work was done to detect changes in spectra caused by different rearing temperatures, spore ages and hosts. The spectra from isolate 742 raised at 16°, 21°, 27° and 32°C are seen in Figures 29-32. Spores raised at 16°C produced 20 bands; those at 21°C, 21; those at 27°C, 18; and those at 32°C, 24. Coefficients of similarity were calculated using the method of Whitney et al. (1968):

$$Cs = \frac{\text{No. of pairs of homologous bands}}{\text{No. of different bands} \quad \text{no. of homologous bands}} \times 100$$

The results of these calculations are seen in Table 3

TABLE 3

COEFFICIENTS OF SIMILARITY (Cs) VALUES

Rearing Temp (Nosema sp. 942)	16°	21°	27°	32°
16°	100	91	100	87
21°	91	100	88	96
27°	100	88	100	87
32°	87	96	87	100

Spectra from spores of isolate 942 raised at 27°C and aged for 5 months were identical to those of fresh spores raised at 32°C (Figures 29-32). The Cs value for fresh and aged spores raised at 27°C was 87. The spectra produced by N. plodiae reared on CSW and Indian meal moths (Plodia interpunctella) were identical and are seen in Figures 31-32.

Diagrammatic representations of the spectra of the 7 Microsporidans tested are seen in Figure 28. Nosema sp. 942 produced 24 bands; Nosema sp. 954, 20; Nosema sp. 960, 18; N. necatrix, 22; N. plodiae, 28; N. algerae, 16; and N. bombycis, 17. Coefficients of similarity were calculated as above and the results are seen in Table 4.

Table

COEFFICIENTS OF SIMILARITY (Cs) VALUES

Isolate	942	954	N. necatrix	960	N. pled	N. al	N. bom
942	100	76	84	75	84	50	50
954	76	100	90	80	65	45	42
N. necatrix	84	90	100	85	73	45	52
960	75	80	85	100	65	45	47
N. plediae	84	65	73	65	100	44	51
N. algerae	50	45	45	45	44	100	43
N. bombycis	50	42	52	47	51	43	100

The Cs values show a high degree of similarity between Nosema spp. 942, 954, 960, N. necatrix and N. plediae. Nosema algerae and N. bombycis were only distantly related to the above group and to each other.

Discussion

Isoelectric focusing

Isoelectric focusing has mainly been employed as a medical diagnostic tool with only Frolin and Glasquez (1972) exploring its taxonomic potential. The results of this study, however, indicate that the method may be useful in determining relationships within the Microsporida. Fowler and Reeves (1974b) reported considerable spectra variation caused by different rearing temperatures, spore ages and hosts when hydrophilic spore proteins were subjected to disc electrophoresis. Isoelectric focusing spectra showed only minor variation related to rearing temperature and spore age (Figures 29-32) and were stable when spores of

N. plodiae were grown in 2 different hosts. Fowler and Reeves (1974b) reported 6-15 bands in each microsporidian spectrum. In the present study approximately twice as many bands (16-28) were seen in each spectrum indicating a higher degree of sensitivity and resolution.

Fowler and Reeves (1974a) found that disc electrophoretic spectra of phenol-acetic acid-water protein extracts may be used to separate species of Microsporida and are not influenced by differences in rearing temperatures, spore ages or hosts. However, they reported a complex of 7 Microsporidans including 3 described species Nosema necatrix, N. plodiae and N. sphingidis which produced identical spectra. In the present study all isolates tested could be separated according to individual spectra including 2 species used by Fowler and Reeves (1974a), N. necatrix and N. plodiae. Thus, isoelectric focusing is more sensitive than disc electrophoresis of PAW extracts.

Where similar species are used, the isoelectric focusing data reported here corroborates the findings of Fowler and Reeves (1974a). Both methods indicate that N. necatrix and N. plodiae are closely related and that Microsporidans forming both Nosema-like monosporea and Thelohania-like octosporea are closely related biochemically as well as morphologically. In addition, Nosema spp. 942 and 960 have been shown to be antigenically similar to N. plodiae.

Nosema algerae and N. bombycis, 2 species which were found to be morphologically similar by Cali (1971), were found to have a low Cs (Table 4), since it has also been shown that the 2 species are serologically dissimilar. It is clear that as discussed by Fowler (1971) classification schemes for the Microsporida based on morphology alone are of dubious value.

The main disadvantages of isoelectric focusing include the cost of the commercial apparatus and the carrier ampholytes. The prepared PAG-plates performed well but the majority of the bands were concentrated within a narrow range (pH 5-8) making analysis difficult. However, Vesterberg (1973) described methods for producing thin layer (2 mm thick) polyacrylamide gels in various pH ranges using combinations of commercially available carrier ampholytes. One such formula has a pH range of 5-8.5 and would be of value for microsporidian proteins. Winogradow et al. (1973) have described a synthesis of carrier ampholytes in the pH range 4-8 which would also be suitable for microsporidian work and has the added advantage of avoiding the expensive commercial products. Similarly, the high cost of the commercially available apparatus could be circumvented by construction of the apparatus developed by Vesterberg (1972).

Although LKB recommends a starting amperage of slightly more than 2 mA/10 cm of gel (50 mA/24 samples) use of this current in the present study invariably resulted in distorted spectra possibly as a result of overheating. Such artifacts were eliminated by using 1 mA/10 cm of gel and taking longer to reach terminal voltage. Vesterberg (1973) adjusted low concentration protein solutions by adding one or more extra saturated pieces of filter paper. Possibly due to the thinness of the gel used in this study, this method resulted in distortion of adjacent spectra. Thus, when using 1 mm thick slabs similar sample volumes must be used. The bands of many superficially different spectra were found upon closer examination to be similar in position but not strength concentrations. In such cases the recording densitometer would be of value in species separation. LKB recommends use of a protein concentration of 1-5mg/ml

in the Multiphor system However, this was found to be much too wide a range for the present work. Most of the samples used in the present study were in the range 4.0-4.5 mg/ml and these gave the best results.

Figure 28. Diagrammatic representation of isoelectric focusing spectra of hydrophilic microsporidian spore proteins. (a) Nosema sp. 942. (b) Nosema sp. 954. (c) Nosema necatrix. (d) Nosema sp. 960. (e) Nosema pieriae. (f) Nosema alpense. (g) Nosema bombycis.

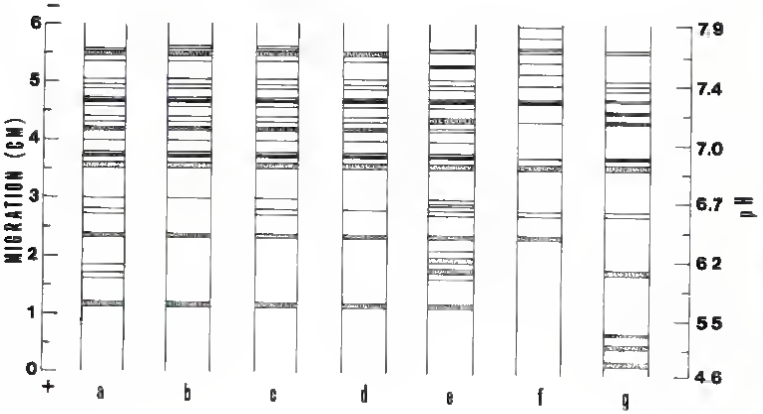
MIGRATION (CM)
−
+
0
1
2
3
4
5
6
a
b
c
d
e
f
g
7.9
7.4
7.0
6.7
6.2
5.5
4.6
pH

Figure 29. Comparison of [illegible] focusing spectra from macrosporidian isolate 942 raised at different temperatures and different aged spores. (a) Isolate 942 raised at 16°C. (b) Isolate 942 raised at 21°C. (c) Isolate 942 raised at 27°C. (d) Isolate 942 raised at 32°C. (e) Same as (a). (f) Isolate 942 raised at 27°C and stored for 5 months before disruption.

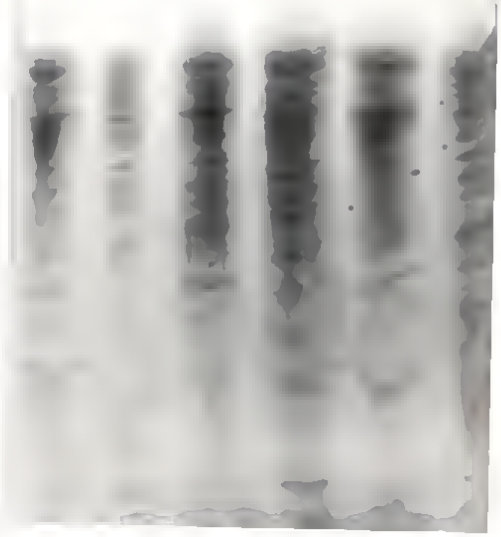

Figure 30. Diagrammatic representation of Figure 29. (a) Isolate 942 raised at 16°C. (b) Isolate 942 raised at 21°C (c) Isolate 942 raised at 27°C (d) Isolate 942 raised at 32°C (e) Same as (a) (f) Isolate 942 raised at 27°C and stored for 5 months before disruption.

MIGRATION (CM)
6
5
4
3
2
1
0
+
a
b
c
d
e
f
pH
7.9
7.4
7.0
6.7
6.2
5.5
4.8

Figure 31. Comparison of isoelectric focusing spectra from Nosema plodiae raised in two different hosts.
(a) Nosema plodiae raised in Plodia interpunctella.
(b) Nosema plodiae raised in Heliothis zea.

Figure 32. Diagrammatic representation of Figure 31. (a) Nosema plediae raised in Pledia interpunctella. (b) Nosema plediae raised in Heliothis zea.

CHAPTER VI

CONCLUSIONS

Of the 4 techniques used in this study, the 2 employing hydrophilic proteins were the most reliable. Using immunodiffusion techniques it was possible to differentiate all 6 of the microsporidian isolates tested and to detect antigenic differences caused by different rearing temperatures. The main disadvantage of serology was the large quantity of spores and length of time required to immunize test animals. This severely limited the number of Microsporidans available for analysis, thus reducing the taxonomic value of serology. The technique would be of more use in biological studies of Microsporida such as investigation of temperature related antigenic differences. Another possible use would be employing fluorescent antibody techniques to locate early stages of infection in host tissue. The main taxonomic value of serology was that it is a well established technique and could be used to gauge the efficacy of other techniques employing hydrophilic protein.

Isoelectric focusing also allowed the separation of all isolates tested and was superior to serology in that it required spores in the 10^9 range as opposed to the more than 10^{11} needed to immunize rabbits. The procedure was much quicker as the time consuming immunization process was eliminated. Due to the large number of bands provided by isoelectric focusing, it was easier to quantify relationships than with the relatively few resulting from immunodiffusion techniques. Immunoelectrophoretic techniques would provide more bands than Ouchterlony plates but require

more spores for immunization so stronger antiserum is required. Future work with isoelectric focusing should be aimed toward a more thorough investigation of the effects of temperature, spore age and host on microsporidian spectra. Once these effects were determined, emphasis could be placed on detecting common patterns in spectra in order to isolate natural groups corresponding to microsporidian species and genera.

Although all microsporidian isolates tested in this study could be separated by the 2 electrophoretic methods employed, Fowler and Reeves (1974a) reported a complex of isolates which had a common spectrum. Therefore, this method appears to be less sensitive than serology or isoelectric focusing. Nevertheless, the method when successfully adapted to micro tubes will be a powerful taxonomic tool as it requires fewer spores than either serology or isoelectric focusing, making the method applicable to a much wider range of Microsporidans. Future work in electrophoresis should be directed towards making the micro technique reliable and producing enough spectra to find common patterns which indicate natural groups. Extracts from disrupted spores will be of the most value in this area as the greater number of bands allows quantification of relationships. Spore coat extracts will be most useful for rapid differentiation of morphologically similar Microsporidans.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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December, 1975

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